

1 Classification: Biological Sciences

2

3

4

5 **Telomere shortening produces an inflammatory environment that**
6 **promotes tumor invasiveness in zebrafish**

7

8 Kirsten Lex^{1*}, Mariana Maia Gil^{1*}, Margarida Figueira¹, Marta Marzullo¹, Bruno Lopes-

9 Bastos^{1,3}, Kety Giannetti¹, Tania Carvalho² and Miguel Godinho Ferreira^{1,3}

10

11 ¹ Instituto Gulbenkian de Ciência, 2781-901 Oeiras, Portugal

12 ² Histology and Comparative Pathology Laboratory, Instituto de Medicina Molecular, 1649-
13 028 Lisbon, Portugal

14 ³ Institute for Research on Cancer and Aging of Nice (IRCAN), INSERM U1081 UMR7284
15 CNRS, 06107 Nice, France.

16

17 * These authors contribute equally in this work

18

19

20

21

22

23

24 Corresponding Author: Miguel-Godinho.FERREIRA@unice.fr

25

26 Keywords: Telomeres, Telomerase, Cancer, Inflammation, Aging

27

28 **Abstract**

29 Cancer incidence increases exponentially with age, when human telomeres are
30 shorter. Similarly, telomerase mutant zebrafish (*tert*) have premature short telomeres and
31 anticipate cancer incidence to younger ages. However, because short telomeres constitute a
32 road block to cell proliferation, telomere shortening is currently viewed as a tumor suppressor
33 mechanism and should protect from cancer. This conundrum is not fully understood. In our
34 current study, we report that telomere shortening promotes cancer in a non-cell autonomous
35 manner. Using zebrafish chimeras, we show increased incidence of invasive melanoma when
36 WT tumors are generated in *tert* mutant zebrafish. *tert* zebrafish show increased levels of
37 senescence (*cdkn2a* and *ink4a/b*) and inflammation (*TNF- α*). In addition, we transferred
38 second generation *tert* blastula cells into WT to produce embryo chimeras. Cells with very
39 short telomeres induced senescence and increased neutrophil numbers in surrounding larval
40 tissues in a non-cell autonomous manner, creating an inflammatory environment. Considering
41 that inflammation is pro-tumorigenic, we transplanted melanoma-derived cells into second
42 generation *tert* zebrafish embryos and observed that tissue environment with short telomeres
43 leads to increased micrometastasis. To test if inflammation was necessary for this effect, we
44 treated melanoma transplants with non-steroid anti-inflammatory drugs and show that higher
45 melanoma invasiveness can be averted. Thus, apart from the cell autonomous role of short
46 telomeres in contributing to genome instability, we propose that telomere shortening with age
47 causes systemic chronic inflammation leading to increased tumor incidence.

48

49

50 **Significance Statement**

51 Cancer incidence increases exponentially in human midlife. Even though mutation
52 accumulation in somatic tissues results in increased tumorigenesis, it is currently not
53 understood how aging contributes to cancer. Telomeres, the ends of eukaryotic linear
54 chromosomes, shorten with each cell division. Here we show that telomere shortening
55 contributes to cancer in a non-cell autonomous manner. Using embryo chimeras of
56 telomerase deficient zebrafish generated from melanoma-prone fish, we show that tumors
57 arise more frequently and become more invasive in animals with shorter telomeres. Telomere
58 shortening gives rise to increased senescence and systemic inflammation. We observed
59 increased melanoma metastasis dissemination in zebrafish larvae with very short telomeres.
60 Thus, telomere shortening similar to human aging, generates a chronic inflammatory
61 environment that increases cancer incidence.

62

63 Introduction

64 Cancer incidence increases exponentially in the mid-decades of human life (1).
65 Although mutations are required to build-up during tumorigenesis, the overall post-
66 reproductive incidence opens the possibility of organism-based causes for the increase of
67 cancer with age. Due to absence of telomerase expression in most somatic tissues, telomeres
68 shorten as we grow older (2). Telomeres constitute the ends of eukaryotic chromosomes and
69 are constituted by repetitive DNA sequences (TTAGGG)_n recognized by a protein complex
70 called shelterin (3). This structure prevents chromosome-ends from being recognized as
71 deleterious DNA double strand breaks while counteracting their slow attrition, resulting from
72 the “end-replication problem” by recruiting telomerase. Humans are born with telomeres
73 between 10-15 kb long (4) and, due to continuous cell divisions, telomeres may reach a
74 critical length. As cell division reaches the Hayflick limit, telomeres are recognized as DNA
75 damage and block cell proliferation either by undergoing senescence or apoptosis (5–7).
76 Since short telomeres block cell division, telomere shortening is considered as a tumor
77 suppressor mechanism by preventing excessive cell proliferation. Indeed, telomerase is
78 frequently re-activated in the majority of cancer cells, allowing for cell immortalization
79 thereby escaping replicative senescence. In line with this idea, anti-telomerase therapies are
80 currently undergoing clinical trials for cancer therapy (8).

81 Countering the tumor suppressor hypothesis, telomere shortening may lead to genome
82 instability, a hallmark of cancer. Because loss of telomere protection results in breakage-
83 fusion-bridge cycles, the ensuing genome instability may contribute for age-dependent
84 tumorigenesis (9). An extreme example of the pro-tumorigenic effect of short telomeres
85 occurs in “telomeropathies”. People carrying mutations in telomerase or related proteins have
86 pathologically short telomeres in early life (10, 11). Despite exhibiting pathologies related to
87 deficiencies in cell proliferation, patients also suffer from an increased cancer risk (12).

88 Similarly, our work on the telomerase mutant zebrafish, which undergoes premature telomere
89 shortening, revealed that they anticipate cancer incidence to early life (13). Even though short
90 telomeres positively correlate with increased tumorigenesis in both humans and zebrafish, it
91 is not yet understood how telomere shortening may lead to cancer.

92 Telomere shortening has consequences beyond the cellular level. As cells approach
93 replicative senescence, DNA damage emanating by short telomeres initiate a cascade of
94 events that expands to the extracellular environment. Senescent cells were shown to release a
95 set of molecules termed senescence-associated secretory phenotype (SASP) (14). SASP was
96 described *in vitro* and is mainly constituted by chemokines, growth factors, extra cellular
97 matrix remodelers and other inflammatory factors, capable of modulating cell environment.
98 These molecules were posteriorly shown to influence the ability of other cells to divide,
99 potentially having a pro-tumorigenic effect (15). Consistently, repeated wounding in
100 zebrafish stimulates inflammatory responses, which were shown to promote cancer
101 progression (16, 17). Therefore, we hypothesize that telomere shortening contribution to
102 tumorigenesis may have a non-cell autonomous component. In aging organisms, cells
103 undergoing replicative senescence would comprise a source of SASP/inflammatory factors
104 creating a pro-tumorigenic environment. In agreement with our hypothesis, population
105 studies have associated the long-term use of anti-inflammatory agents (acetylsalicylic acid)
106 and a reduction risk of several cancers (18–20).

107 Here we show that tissues containing cells with short telomeres promote increased
108 cancer incidence in a non-cell autonomous manner. Using chimeric zebrafish, we observed
109 that telomerase-proficient melanocytes expressing HRAS give rise to more melanoma tumors
110 when surrounded by *tert* mutant cells. Melanomas developed in this environment exhibited
111 high invasiveness as observed by histopathology. In agreement, using zebrafish tumor
112 transplants, we show that HRAS melanoma cells expand faster when injected into second-

113 generation (G2) *tert* mutant larvae. Both adult G1 *tert* and G2 *tert* larvae have higher levels
114 of senescence and SASP/inflammation. G2 *tert* cells injected into WT embryos stimulate
115 senescence and inflammation in a non-cell autonomous manner. Chemical inhibition of
116 inflammation in G2 *tert* embryos rescued the invasiveness capacity of melanoma cells. Thus,
117 cells with short telomeres are capable of inducing senescence and inflammation, creating a
118 pro-tumorigenic environment that results in higher cancer invasiveness.

119

120 **Results**

121 ***tert* mutant environment causes higher tumor incidence in a non-cell autonomous** 122 **manner**

123 Similar to mammals, zebrafish tumor microenvironment (TME) modulates cancer
124 behavior (21, 22). Tumors may be inhibited or enhanced as a consequence of the dynamic
125 crosstalk between cancer and surrounding cells. We, therefore, asked what were the effects of
126 a TME with short telomeres on emergent tumors.

127 In order to study the non-cell autonomous effects of TME telomere shortening in
128 cancer, we wanted to separate telomerase expression of pre-cancer cells from their
129 surrounding tissues and, for this purpose, we generated chimeric zebrafish using early-
130 developmental embryo transplants. We used a melanoma zebrafish model (*mitfa*:HRAS)
131 developed by the Hurlstone lab that exhibits full penetrance by 3 months of age (23). We
132 chose this model since it did not require an initial *tp53* dysfunction to form tumors and we
133 had previously shown that loss of p53 function rescues *tert* zebrafish mutants (24). Blastula
134 cells from donor embryos capable of giving rise to melanoma were transplanted into WT or
135 *tert*^{-/-} recipients (Fig 1A). In addition, recipient embryos had a *casper* genetic background
136 (*mitfa*^{w2/w2}; *mpv17*^{a9/a9}), and lacked the ability to produce melanocytes. Consequently, all

137 melanoma could only arise from donor cells. Embryo chimeras then were allowed to grow
138 into adulthood and studied for tumor incidence. As expected, we observed the development
139 of melanoma lesions, typically in the anal fin region of both WT and *tert*^{-/-} recipient fish
140 (Fig. 1B). However, by 30 weeks, a time when *tert*^{-/-} associated lethality is still low (<20%),
141 20% of WT chimeras developed tumors, while ca. 50% of *tert*^{-/-} chimeras exhibited
142 melanoma (Fig. 1C, $p < 0.05$). Thus, we found that *tert*^{-/-} recipients significantly increased
143 tumor incidence by ca. 2-fold (Hazard ratio after Mantel-Haenszel calculation: 2.0 when
144 compared with WT fish).

145 A possible explanation for the observed differences of tumor development in a WT
146 vs. *tert*^{-/-} environment is cell competition. Wildtype tumor-prone cells could be fitter and
147 more efficient in outcompeting *tert* mutant recipient cells, possibly due to higher proliferation
148 rates. Thus, fitter donor cells could produce higher number of melanocytes expressing HRAS
149 in *tert* mutant recipients and, subsequently, lead to a higher tumor incidence. To test this
150 hypothesis, we quantified the number of melanocytes at two stages of embryo development at
151 3- and 11-days post-fertilization (dpf) in both *tert* mutant and WT recipients. Contrary to our
152 hypothesis, we observed no significant increase in the number of melanocytes in *tert*^{-/-}
153 recipients as compared to WT during developmental stages (Supplementary Figure 1A-B). In
154 case growth differences would only be visible at later stages, we quantified the surface area
155 covered by the melanocytic lesions in adult animals. Percentage of pigmentation was
156 quantified for WT and *tert*^{-/-} zebrafish (Supplementary Figure 1C-D). Similar to the results
157 obtained in larvae, although there was variation between individuals, we did not observe
158 significant differences when comparing host genotypes. Together, our data indicates that a
159 *tert* mutant TME increases tumor incidence in a non-cell autonomous manner, suggesting that
160 telomere shortening has a systemic role in cancer beyond the one described in genome
161 stability.

162

163 **Tumors progress faster in *tert* mutant TME**

164 Among the hallmarks of cancer, one qualitative difference between cancers relies on
165 the capacity to invade different tissues. Zebrafish chimeras bearing melanoma were analyzed
166 by histopathology and ranked according to their staging and invasiveness. Overall, 84% of
167 samples (N=43) that were macroscopically defined as tumors were confirmed as malignant
168 tumors in histopathological analysis (Fig. 2 A-C). The remaining samples were staged as
169 benign tumors or melanosis. The large majority of tumors in *tert*^{-/-} recipients were invasive
170 (80%, N=10; Fig. 2 B). In comparison, only 22% of tumors exposed to a wildtype
171 environment (N=9) were determined as invasive (Fig. 2 B). A similar result was found when
172 malignant tumors were scored for the presence of cellular atypia. Cellular atypia describes
173 cytologic structural abnormalities and is a marker for more transformed cancers and more
174 advanced staging (25). Whereas 71% of tumors in a *tert*^{-/-} environment (N=7) exhibited
175 moderate levels of cellular atypia (Fig. 2 C), all tumors in WT recipients showed low levels
176 (N=5). These results indicate that melanoma developed in *tert*^{-/-} recipients progress faster,
177 reaching advanced stages faster and becoming more invasive, suggesting that TME telomere
178 shortening not only increase tumor incidence but its progression.

179

180 **Zebrafish melanoma transplants are more invasive in *tert* mutant larvae**

181 We and others have shown that injection of tumor cells in zebrafish larvae constitutes
182 an assay to study invasiveness capacity of cancer cells (26, 27). This constitutes a simpler
183 assay and allows for more expedite manipulations while being amenable to chemical studies.

184 In order to confirm that *tert*^{-/-} TME promotes tumor invasiveness, we injected
185 melanoma cells derived from HRAS tumors into 2dpf WT and *tert*^{-/-} larvae (Fig. 3A). To
186 ensure that these fish would possess cells with critically short telomeres, we used second

187 generation *tert*^{-/-} (G2 *tert*^{-/-}) resulting from an in-cross of young adult *tert*^{-/-} zebrafish. In
188 contrast to G1 *tert*^{-/-} derived from heterozygous parents, G2 *tert*^{-/-} embryos possess very
189 short telomeres and a high mortality with an average longevity of ~12days (28, 29). We
190 dissected melanomas from HRAS tumors expressing GFP (see Methods) and injected cells
191 into the blood circulation of 2dpf larvae. Injected melanoma cells preferentially accumulate
192 in the tail region from where, depending on their invasiveness capacity, disseminate to
193 neighboring tissues (Fig. 3B). Injected larvae were individually followed over time and the
194 area occupied by GFP cells was quantified (Fig. 3B).

195 If an environment with short telomeres promotes tumor invasiveness, then injected
196 melanoma cells should disseminate more when injected in G2 *tert*^{-/-} when compared to WT
197 larvae. We quantified the GFP-area at 1, 4 and 7 days-post injection (Fig. 3C). We calculated
198 the linear regression between the 3 time-points and obtained a progression slope for the
199 expansion of each grafted melanoma (N=31). We observed that *tert*^{-/-} recipients allowed for
200 a more accentuated progression than the WT ones (Fig. 3D). Thus, our results using tumor
201 transplants indicate that melanoma cells disseminate faster in G2 *tert*^{-/-} than WT larvae,
202 suggesting that telomere shortening in aging individuals could promote tumor progression in
203 a non-cell autonomous manner.

204

205 **G2 *tert*^{-/-} cells are senescent and inflammatory and capable of modulating their**
206 **surrounding environment.**

207 Telomere shortening is responsible for replicative cell senescence in human cultured cells
208 (30). Accordingly, we expected that *tert*^{-/-} zebrafish would present increased levels of
209 senescence. Using RT-qPCR for specific genes, we quantified the levels of senescence in
210 *tert*^{-/-} 9month-old adult tissue (intestine) and 4dpf G2 *tert*^{-/-} larvae (whole). As expected, the
211 senescence markers *ink4a/b* (p15/16) and *cdkn1a* (p21) levels were significantly higher in

212 both G1 *tert*^{-/-} adults and G2 *tert*^{-/-} larvae than in WT controls (Fig. 4A-B). In addition,
213 using the SA- β -Gal assay, we confirmed higher levels of senescence localized primarily in
214 the head and notochord of G2 *tert*^{-/-} larvae (Fig. 4D).

215 Senescent cells were shown to secrete a set of molecules, known as SASP, mainly
216 composed of inflammatory factors (14, 15). Therefore, we asked if *tert*^{-/-} zebrafish present
217 signs of inflammation. We measured expression levels of TNF- α , one of the main cytokines
218 expressed during an inflammatory response, by RT-qPCR. Indeed, both G1 *tert*^{-/-} adults and
219 G2 *tert*^{-/-} larvae showed elevated levels of TNF- α when compared to WT (Fig. 4C).
220 Interestingly, undisturbed 9month-old WT zebrafish exhibit higher levels of TNF- α than
221 4day-old larvae, suggesting that aging animals may respond similarly to young *tert*^{-/-}
222 mutants. Together, our results suggest that telomere shortening in zebrafish results in
223 increased senescence and inflammation.

224 Given the nature of the responses, we wondered if these observations originated from
225 cell-autonomous effects of *tert*^{-/-} cells dispersed through the body or if *tert*^{-/-} cells could
226 modulate their extracellular environment *in vivo* and generate a systemic response. To test if
227 short telomere *tert*^{-/-} cells modulate their extracellular environment, we transplanted GFP-
228 labelled G2 *tert*^{-/-} cells during early-development into WT recipient embryos, thereby
229 generating larvae chimeras (Fig. 4E). Even though we transferred few G2 *tert*^{-/-} cells into
230 developing embryos (<1% as measured by FACS of desegregated embryos at 4dpf), they
231 were sufficient to increase overall SA- β -Gal levels (Fig. 4E). Interestingly, we observed a
232 similar pattern of SA- β -Gal staining in these chimeras as in G2 *tert*^{-/-} larvae (N=22) at the
233 same stage of 4dpf (compare Fig. 4D with 4E). These results suggest that cells derived from
234 G2 *tert*^{-/-} embryos are capable of inducing senescence in a non-cell autonomous manner, thus
235 constituting an example of paracrine SASP.

236 Since senescent cells secrete pro-inflammatory molecules, we asked if G2 *tert*^{-/-} cells

237 with short telomeres could create an inflammatory environment in newly generated chimeras.
238 To test this, we generated similar embryo chimeras in Tg(*mpx:GFP*) recipient zebrafish that
239 carry GFP-labelled neutrophils (31). As before, we injected both WT and G2 *tert*^{-/-} cells
240 from embryos at blastula stage into Tg(*mpx:GFP*) recipient embryos of the same stage and
241 observed its effects in 4dpf larvae (Fig. 4F, right). Whereas WT cells generated zebrafish
242 larvae (N=33) with similar numbers of neutrophils as un-injected embryos, Tg(*mpx:GFP*)
243 chimeras carrying G2 *tert*^{-/-} cells (N=25) exhibited higher numbers of neutrophils (Fig. 4F,
244 p=0.0075). Thus, since these innate immune cells are key to inflammatory responses, G2 *tert*^{-/-}
245 *tert*^{-/-} cells give rise to a systemic inflammatory environment. Together, our results indicate that
246 telomerase deficient zebrafish undergo senescence and produce an inflammatory state.
247 Moreover, we show that this effect is non-cell autonomous with *tert*^{-/-} cells impacting the
248 surrounding tissues modulating their environment, creating a senescent and inflammatory
249 environment.

250

251 **Chemical inhibition of inflammation rescues melanoma dissemination in the G2 *tert*^{-/-}**
252 **mutant larvae.**

253 Inflammation can induce transformed cell growth (32). In zebrafish, PGE₂ produced
254 by innate immune cells via the COX-2 pathway was shown to act as key growth factor at the
255 earliest stages of tumor progression (16, 33). We hypothesized that the inflammatory
256 environment induced by *tert*^{-/-} cells could underlie the enhanced melanoma invasiveness
257 observed in *tert* mutant zebrafish. To test this hypothesis, we treated the previously generated
258 zebrafish melanoma larvae allografts with non-steroid anti-inflammatory drugs (NSAIDs):
259 Aspirin (COX-1 and 2 inhibitor) and Celecoxib (COX-2 specific inhibitor). As previously,
260 we measured melanoma invasiveness by quantifying the GFP area at consecutive timepoints
261 upon melanoma cell injections (1, 4 and 7 dpi). Both WT and G2 *tert*^{-/-} recipients were kept

262 in embryo medium containing Aspirin (30 μ M) or Celecoxib (25 μ M) for the duration of the
263 experiment. As previously, we calculated a progression slope of tumor cells per transplanted
264 zebrafish and compared treated vs. untreated larvae (Fig. 5A-B). As previously, control
265 groups showed an increased invasiveness of melanoma cells when transplanted into *G2 tert*^{-/-}
266 (N=31) than in WT larvae (N=32) (Fig. 5D, p= 0.0205). However, upon NSAID treatment,
267 the increased invasion capacity of HRAS cells in *G2 tert*^{-/-} larvae (N=20) decreased to WT
268 levels (N=19) (Fig. 5C-D, Aspirin p=0.7897; Celecoxib: p= 0.1605). Together, our result
269 suggests that the inflammatory environment induced by *tert*^{-/-} cells promotes melanoma
270 invasiveness via the COX-2 pathway. We showed an increase of innate immune cells in
271 larvae containing telomerase deficient cells (Fig. 4F). Thus, in agreement with previous
272 studies (16, 33, 34), we propose that neutrophils, by producing larger amounts of
273 prostaglandins, may enhance melanoma invasiveness.
274

275 Discussion

276 Studies on how telomerase affects tumorigenesis have focused primarily on the cell-
277 autonomous role of telomere shortening in cancer cells (9). Indeed, telomerase is reactivated
278 in the majority of cancer cells promoting cancer development. Consistently, telomerase
279 promoter mutations that result in increased telomerase expression are now recognized as one
280 of the most common alterations in cancer (35). However, cancer incidence increases
281 exponentially in the mid-ages of human life, a time when telomeres are shorter (1, 2). In our
282 current study, we attempted to understand why cancer incidence increases when telomeres
283 are shorter. Apart from the recognized cell autonomous role in tumor suppression, we
284 propose that telomere shortening affects tumorigenesis in a non-cell autonomous manner. As
285 an organism grows older, increasing numbers of cells with short telomeres modulate their
286 surrounding environment creating a pro-inflammatory milieu that promotes tumorigenesis.

287 Using zebrafish embryo chimeras and cancer transplants, we show that incidence of
288 melanoma is not only higher but progresses faster in animals deficient for telomerase. Both
289 adult G1 *tert*^{-/-} and G2 *tert*^{-/-} embryos have shorter telomeres and mount DNA damage
290 responses that stabilize p53 leading to premature aging and death (13, 28, 29). Indeed,
291 mutations in *tp53* rescue the severity of both *tert*^{-/-} models, allowing for prolonged survival.
292 Spontaneous cancer in zebrafish, as in humans, is an age-associated disease that quickly rises
293 upon decline of reproductive age (13, 36). Like other age-related phenotypes, spontaneous
294 tumors in *tert*^{-/-} zebrafish are accelerated to younger ages, while remaining similar in
295 incidence and spectrum. Indeed, telomerase deficiency and telomere shortening in zebrafish
296 do not appear to restrain tumorigenesis. Rather, they promote early cancer incidence denoting
297 a systemic role in their effects. Similarly, humans with deficiencies in telomerase and
298 premature telomere shortening show an increased cancer predisposition at younger ages (12).
299 Thus, beyond preventing uncontrolled cell proliferation, absence of telomerase and telomere

300 shortening appear to have a systemic role impairing health status and resistance to disease.

301 How could telomere shortening in surrounding tissues lead to increased incidence of
302 cancer? We observed that *tert*^{-/-} zebrafish present high levels of senescence. Studies *in vitro*
303 revealed that senescent cells secrete SASP, composed by several inflammatory factors (14,
304 15). In agreement, we observed that *tert*^{-/-} zebrafish present high levels of *cdkn1a* and *ink4ab*
305 senescence genes and TNF- α , a cytokine involved in systemic inflammation. Moreover, G2
306 *tert*^{-/-} cells are capable of inducing systemic senescence and inflammation in a non-cell
307 autonomous manner. This data constitutes a strong indication that cells with short telomeres
308 are a source of paracrine SASP *in vivo*. However, similar to other studies in zebrafish (37),
309 we were unable to detect other typical SASP cytokines in *tert*^{-/-} zebrafish larvae, such as IL6
310 and IL10. The main *in vivo* SASP molecules are yet to be identified in zebrafish.

311 Consistent with higher levels of inflammatory cytokines, G2 *tert*^{-/-} cells containing
312 critically short telomeres can modulate their environment by increasing the number of
313 neutrophils. An increase in innate immune cells is characteristic of an inflammatory
314 environment which can be tumorigenic. Human skin cancers have been shown to increase
315 upon repeated injury and ulcers of previous lesions (38). In zebrafish, Feng *et al.* showed that
316 preventing the recruitment of innate immune cells reduced the growth of HRAS^{G12V}-
317 transformed cells (16). Moreover, PGE2 produced by immune cells were shown to constitute
318 a source of supportive signals for cancer cell growth. In line with this study, we observed a
319 reduction of melanoma invasiveness with anti-inflammatory treatment, such as Aspirin and
320 Celecoxib. Thus, our results suggest that G2 *tert*^{-/-} cells with short telomeres promote the
321 tumor invasiveness through the COX-2 pathway.

322 Collectively, our data indicates that an environment with short telomeres promotes
323 tumorigenesis in a non-cell autonomous manner and increases the invasiveness capacity of
324 melanoma cells. Apart from the recognized cell autonomous role in blocking uncontrolled

325 cell division, telomere shortening and senescence may have a second, perhaps, antagonistic
326 pleiotropic consequence of causing local tissue damage and chronic inflammation. Thus, we
327 propose that telomere shortening during aging gives rise to a systemic inflammatory
328 environment. Chronic inflammation may be part of the mechanism whereby telomere
329 shortening leads to increase tumorigenesis with age. Indeed, whereas chronic inflammation
330 was shown to be a contributing factor in several cancers, immunosuppression leads to
331 increase the risk for certain tumors (39, 40). Epidemiology studies associate the long-term
332 dosage of Aspirin with a reduced incidence of certain types of cancer (18–20). Interestingly,
333 this effect is more pronounced with increased age of the population. Reverting telomere
334 shortening in animal models that possess short telomeres, such as the zebrafish, will
335 conclusively test the idea if repression of telomerase promotes cancer in aging.

336

337

338 **Materials and Methods**

339 **Ethics statement**

340 All Zebrafish work was conducted according to National Guidelines and approved by the
341 Ethical Committee of the Instituto Gulbenkian de Ciência and the DGAV (Direcção Geral de
342 Alimentação e Veterinária, Portuguese Veterinary Authority).

343 **Zebrafish maintenance and standard techniques**

344 Zebrafish were maintained in accordance with Institutional and National animal care
345 protocols. For normal line maintenance embryos were collected from crosses and kept in E3
346 embryo medium (5.0mM NaCl, 0.17mM KCl, 0.33mM CaCl, 0.33mM MgSO₄, 0.05%
347 methylene blue, pH 7.4) at 28°C on a 14h light/10h dark cycle. At 5-6dpf larvae were
348 transferred into a recirculating system at 28°C, with a 14h light/10h dark cycle.

349 For anesthesia, fish were immersed into tricaine methane sulfonate solution at 168µg/L

350 (MS222 Sigma) and after the procedure placed back into system water. Their recovery was
351 monitored until they regained normal swimming ability. Tricaine methane sulfonate was used
352 at high concentration (200mg/L) to sacrifice fish. Larvae (until 7dpf) were sacrificed by
353 placing them in ice-cold water for longer than 20min.

354 **Transgenic and mutant zebrafish lines**

355 The telomerase mutant line *tert*^{AB/hu3430} generated by N-Ethyl-N-nitrosourea (ENU)
356 mutagenesis (Utrecht University, Netherlands; Wienholds, 2004), has a T→A point-mutation
357 in the *tert* gene and is available at the ZFIN repository, ZFIN ID: ZDB-GENO-100412-50,
358 from the Zebrafish International Re-source Center – ZIRC. The *tert*^{hu3430} mutation was
359 combined by genetic crossing in a *casper* background strain leading to the complete lack of
360 pigmentation (41). For maintenance of this line, *casper; tert*^{AB/hu3430} was continuously
361 outcrossed to *casper; tert*^{+/+}. All recipient embryos used for the generation of HRAS
362 chimeras, *tert*^{hu3430/hu3430} homozygous mutants (referred to as *tert*^{-/-}) as well as their WT
363 siblings were obtained by incrossing *casper; tert*^{AB/hu3430} animals. Donor embryos carry two
364 different transgenes: Tg(*mitfa*:HRAS^{G12}-*mitfa*:GFP; β -*actin*:mGFP)). They express GFP and
365 a mutated and oncogenic version of human HRAS under a melanocyte-specific promoter
366 *mitfa* causing strong hyperpigmentation and the formation of melanoma (23). We used a
367 Tg(β -*actin*:mGFP) line with ubiquitous expression of membrane bound-GFP (mGFP) (42),
368 since *mitfa*:GFP is only visible upon melanocyte development.

369 **Generation of zebrafish chimeras**

370 Both donor and recipient embryos were manually dechorionated using forceps (not earlier
371 than 16 cell-stage). Dechorionated embryos were maintained in transplant-media (14.97mM
372 NaCl; 503 μ M KCl; 1.29mM CaCl₂ · 2H₂O; 150.63 μ M KH₂PO₄; 50 μ M Na₂HPO₄;
373 994.04 μ M MgSO₄ · 7H₂O) with penicillin/streptomycin (100U/ml penicillin and 100 μ g/ml
374 streptomycin) in agarose-coated plates until 48hpf after which they were transferred into E3

375 embryo medium in non-coated petri dishes. Cell transfer from donor to recipient embryo was
376 performed at blastula-stage using a hydraulic, manual microinjector (CellTram® vario,
377 Eppendorf) with needles pulled from capillaries (TW100-4, World precision instruments,
378 with a tip clipped off and polished of inner diameter at the tip 40-45µm) using a fluorescent
379 stereoscope (Leica M205FA). Labelled donor cells (GFP+) were taken from
380 *Tg(mitfa:HRAS^{G12}-mitfa:GFP; β-actin:mGFP)* embryos and injected into recipient embryos.
381 Cells were taken up by gentle suction directly at the blastula surface and released by injecting
382 into the blastula of the recipient without ever harming the yolk cell. To increase the
383 likelihood of transferring neural crest progenitors for tumor studies in adult animals, cells
384 were typically taken from 3-5 spots at different sides of the donor embryo, all aligned
385 midway between animal pole and yolk cell. Directly upon transfer, around 5% (estimation) of
386 cells in a chimeric embryo were donor-derived. Single donor embryos served usually for
387 various recipients (up to four), but one recipient never received cells from mixed donors.
388 Upon cell transfer embryos were kept at low density (max 50 per plate) at 28°C and cleaned
389 daily.

390 **Selection of Chimeras to grow and tumor assessment**

391 All animals included in this study were screened for a normal phenotype, presence of
392 melanocytes and presence of GFP-positive donor cells. This screening was done under light
393 anesthesia (84µg/L tricaine methane sulfonate MS222 in E3 embryo medium, 50% of
394 standard concentration) under a Fluorescence stereomicroscope (Leica M205FA).
395 Tumor appearance was assessed weekly and macroscopically. Individual animals were scored
396 for the onset of a vertical growth phase, the presence of an outgrowth in any direction.
397 Subsequently, most animals were analyzed by histopathology to confirm tumor formation and
398 the state of invasiveness.

399 **Fish preservation for histology**

400 When possible, fish were food-deprived for 24h prior to processing. After sacrificing,
401 pictures of each fish were taken from both sides, both with a regular camera and at the
402 fluorescent stereoscope (Leica M205FA) to save information about the gross distribution of
403 pigmentation and chimeric (GFP+) cells. Animals were fixed in 10% neutral buffered
404 formalin for 72h at room temperature and decalcified in 0.5M EDTA for 48h. Whole fish
405 were paraffin embedded and 3µm transversal cuts were done from 5-8 regions of the fish
406 (depending on size). Cuts were stained with haematoxylin and eosin and analyzed by
407 histopathology. A total of N=18 animals was analyzed (9 WT and 9 *tert*^{-/-} recipients).

408 **Melanoma cell transplants into 2dpf larvae**

409 Melanoma cells were derived from Tg(*mitfa*:*HRAS*^{G12}-*mitfa*:*GFP*) zebrafish tumors. To
410 obtain the tumor cells fish were first sacrificed with tricaine 25x and the tumor was dissected
411 with a regular scalpel and scissors. To dissociate the tumor, the mass of cells was dissected in
412 small pieces, placed in a tryplE solution and pipetted up and down. Enzymatic reaction was
413 stop with the addition of FBS (10% of total volume). Solution was filtered (70µm filter) and
414 spun down at 1700 rpm for 5 min. The pellet was re-suspended in PBS calcium/magnesium
415 free and then washed in culture medium with PBS (DMEM + 10% FBS). The final solution
416 was approximately 1x10⁷ cells/mL and was obtained by removing as much as possible
417 supernatant in the last centrifugation.

418 Melanoma cells were injected into the circulation of 2dpf larvae with a microinjection
419 apparatus and needles were pulled from capillaries (TW100-4, World precision instruments).
420 Transplanted larvae were kept overnight at 28°C in embryo media. In the following day
421 larvae are screened for the presence of GFP positive cells in the tail region and only those
422 continue in the experiment. Pictures were taken at 1, 4 and 7 days-post injection using a
423 fluorescent stereoscope (Leica M205FA). Transplanted larvae were kept in individual wells
424 of a 6 well-plate to allow individual tracking of melanoma progression. Control (E3) or

425 treatment (Aspirin - 30 μ M; Celecoxib – 25 μ M dissolved in DMSO) media were replaced
426 daily. These experiments were repeated 2-3 times. GFP area was quantified using the
427 Analyze Particles tool of imageJ 1.52i software.

428 **Statistical analysis**

429 Statistical analysis was done with the Software GraphPad Prism 6. Comparisons of two
430 different points were done by unpaired t-test. For the G1 HRAS chimeras, comparison over
431 time (for at least 2 timepoints) was performed by Two-way RM ANOVA. Tumor onset over
432 time was compared using a Log-rank (Mantel-Cox) test. A critical value for significance of
433 $p < 0.05$ was used throughout the study. For the larvae transplants, trend lines of GFP area
434 between the three time-points (1, 4 and 7 days-post injection) per transplanted zebrafish were
435 calculated using Microsoft Excel 2010 software. Slopes averages were compared between
436 each two conditions using unpaired t-test with the Software GraphPad Prism 6.

437 **Senescence-associated β -galactosidase assay**

438 β -galactosidase assay was performed as previously described (43). Briefly, sacrificed
439 zebrafish larvae were fixed over-night in 4% paraformaldehyde in PBS at 4°C and then
440 washed three times for 1 h in PBS-pH 7.4 and for a further 1 h in PBS-pH 6.0 at 4°C. β -
441 galactosidase staining was performed for 10h at 37°C in 5 mM potassium ferrocyanide, 5
442 mM potassium ferricyanide, 2mM MgCl₂ and 1 mg/ml X-gal, in PBS adjusted to pH 6.0.
443 After staining, larvae were washed three times for 5 minutes in PBS pH 7, observed and
444 photographed using a bright filter stereoscope (Leica M205FA).

445 **Real-time quantitative PCR**

446 4dpf larvae were sacrificed, immediately snap-frozen in liquid nitrogen and collected in
447 Eppendorf tube, minimum 10 larvae each. RNA extraction was performed using a RNeasy
448 extraction kit (Qiagen, UK # 50974134). Briefly, larvae were smashed in RLT lysis buffer
449 (provided by the kit) and the extract was washed and RNAs isolated through RNA binding

450 column and eluted in dH₂O RNase-free, according to manufacture procedures. Quality of
451 RNA samples was assessed through BioAnalyzer (Agilent 2100, CA, USA). Retro-
452 transcription into cDNA was performed using a RT-PCR kit NZY First-Strand cDNA
453 Synthesis Kit # MB12501 (NZYtech). Quantitative PCR (qPCR) was performed using iTaq
454 Universal SYBR Green Supermix # 1725125 (Bio-Rad) and an ABI-QuantStudio 384
455 Sequence Detection System (Applied Biosystems, CA, USA). qPCRs were carried out in
456 triplicate for each cDNA sample. Relative mRNA expression was normalized to rpl13 mRNA
457 expression using the DCT method. Primer sequences are listed in Table S1.

458

459 **Table S1 – List of primers used in RT-qPCR expression analysis and *tert* genotyping.**

Gene name	Primer sequences
<i>p15/16</i>	forward – 5' GGATGAACTGACCACAGCAGCA 3' reverse – 5' CGGCTGCGGAAAGAGTCTCAG 3'
<i>p21</i>	forward – 5' ATGCAGCTCCAGACAGATGA 3' reverse – 5' CGCAAACAGACCAACATCAC 3'
<i>TNFα</i>	forward – 5' AGGCAATTTCACTTCCAAGGC 3' reverse – 5' GGTCCTGGTCATCTCTCCAGT 3'
<i>RPL13</i>	forward – 5' TTCACCACCACAGCCGAAAGA 3' reverse – 5' TACCGCAAGATTCCATACCCA 3'

460

461 **Acknowledgements**

462 We thank members of the Telomeres and Genome Stability Laboratory for helpful
463 discussions. We are grateful to Yi Feng (U of Edinburgh), Thiago Carvalho (Fundação
464 Champalimaud) and Leonor Saúde (Instituto de Medicina Molecular) for critically reading
465 our manuscript. We thank the Instituto Gulbenkian de Ciência histology unit and the Fish

466 Facility for excellent animal care. KL was a recipient of a Portuguese Fundac o para a
467 Cie ncia e a Tecnologia (FCT) fellowship SFRH/BD/52173/2013. This work was supported
468 by the FCT (PTDC/BIM-ONC/3402/2014 and PTDC/SAU-ONC/116821/2010) and the
469 Howard Hughes Medical Institute grants received by MGF.

470

471 **Author contributions**

472 Conceived and designed the experiments: MGF KL MF and MMG. Performed the
473 experiments: KL MMG MF MM BLB KG. Analysed the data: KL MMG MF TC MGF.
474 Contributed reagents/materials/analysis tools: KL MMG MF MM BLB KG TC MGF. Wrote
475 the paper: MGF KL MMG.

476

477 **References**

- 478 1. Parkin DM, Pisani P, Ferlay J (1999) Global cancer statistics. *CA Cancer J Clin*
479 49(1):33–64.
- 480 2. Aubert G, Lansdorp PM (2008) Telomeres and Aging. *Physiol Rev* 88(2):557–579.
- 481 3. Palm W, de Lange T (2008) How shelterin protects mammalian telomeres. *Annu Rev*
482 *Genet* 42:301–334.
- 483 4. Samassekou O, Gadjji M, Drouin R, Yan J (2010) Sizing the ends: Normal length of
484 human telomeres. *Ann Anat - Anat Anzeiger* 192(5):284–291.
- 485 5. Zhang X, Mar V, Zhou W, Harrington LA, Robinson MO (1999) Telomere shortening
486 and apoptosis in telomerase-inhibited human tumor cells. *Genes & Dev*
487 13(18):2388–2399.
- 488 6. Fagagna F d’Adda di, et al. (2003) A DNA damage checkpoint response in telomere-
489 initiated senescence. *Nature* 426(6963):194–198.
- 490 7. Lechel A, et al. (2005) The cellular level of telomere dysfunction determines induction
491 of senescence or apoptosis in vivo. *EMBO Rep* 6(3):275–281.
- 492 8. Harley C (2008) Telomerase and cancer therapeutics. *Nat Rev Cancer* 8(3):167–179.
- 493 9. Shay JW, Wright WE (2011) Role of telomeres and telomerase in cancer. *Semin*
494 *Cancer Biol* 21(6):349–353.
- 495 10. Armanios M, Blackburn EH (2012) The telomere syndromes. *Nat Rev Genet*
496 13(10):693–704.
- 497 11. Holohan B, Wright WE, Shay JW (2014) Telomeropathies: An emerging spectrum
498 disorder. *J Cell Biol* 205(3):289–299.
- 499 12. Alter BP, Giri N, Savage SA, Rosenberg PS (2009) Cancer in dyskeratosis congenita.
500 *Blood*. doi:10.1182/blood-2008-12-192880.
- 501 13. Carneiro MC, et al. (2016) Short Telomeres in Key Tissues Initiate Local and

- 502 Systemic Aging in Zebrafish. *PLOS Genet* 12(1):e1005798.
- 503 14. Coppé J-P, et al. (2008) Senescence-Associated Secretory Phenotypes Reveal Cell-
504 Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLoS*
505 *Biol* 6(12):e301.
- 506 15. Coppé J-P, Desprez P-Y, Krtolica A, Campisi J (2010) The Senescence-Associated
507 Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu Rev Pathol Mech*
508 *Dis*. doi:10.1146/annurev-pathol-121808-102144.
- 509 16. Feng Y, Renshaw S, Martin P (2012) Live Imaging of Tumor Initiation in Zebrafish
510 Larvae Reveals a Trophic Role for Leukocyte-Derived PGE2. *Curr Biol* 22(13):1253–
511 1259.
- 512 17. Feng Y, Martin P (2015) Imaging innate immune responses at tumour initiation: New
513 insights from fish and flies. *Nat Rev Cancer*. doi:10.1038/nrc3979.
- 514 18. Cao Y, et al. (2016) Population-wide Impact of Long-term Use of Aspirin and the Risk
515 for Cancer. *JAMA Oncol* 2(6):762.
- 516 19. Gamba CA, et al. (2013) Aspirin is associated with lower melanoma risk among
517 postmenopausal Caucasian women: The Women’s Health Initiative. *Cancer*.
518 doi:10.1002/cncr.27817.
- 519 20. Rothwell PM, et al. (2011) Effect of daily aspirin on long-term risk of death due to
520 cancer: analysis of individual patient data from randomised trials. *Lancet*
521 377(9759):31–41.
- 522 21. Kim IS, et al. (2017) Microenvironment-derived factors driving metastatic plasticity in
523 melanoma. *Nat Commun* 8(1):14343.
- 524 22. Tobia C, Gariano G, De Sena G, Presta M (2013) Zebrafish embryo as a tool to study
525 tumor/endothelial cell cross-talk. *Biochim Biophys Acta - Mol Basis Dis*.
526 doi:10.1016/j.bbadis.2013.01.016.
- 527 23. Michailidou C, et al. (2009) Dissecting the roles of Raf- and PI3K-signalling pathways
528 in melanoma formation and progression in a zebrafish model. *Dis Model & Mech*
529 2(7–8):399–411.
- 530 24. Henriques CM, Carneiro MC, Tenente IM, Jacinto A, Ferreira MG (2013) Telomerase
531 is required for zebrafish lifespan. *PLoS Genet* 9(1):e1003214.
- 532 25. AI B, C C (2007) TUMOR CELL MORPHOLOGY. *Comparative Oncology*.
- 533 26. Fior R, et al. (2017) Single-cell functional and chemosensitive profiling of
534 combinatorial colorectal therapy in zebrafish xenografts. *Proc Natl Acad Sci U S A*
535 114(39). doi:10.1073/pnas.1618389114.
- 536 27. Moore JC, Langenau DM (2016) Allograft Cancer Cell Transplantation in Zebrafish.
537 *Advances in Experimental Medicine and Biology*, pp 265–287.
- 538 28. Anchelin M, et al. (2013) Premature aging in telomerase-deficient zebrafish. *Dis*
539 *Model & Mech* 6(5):1101–1112.
- 540 29. Henriques CM, Carneiro MC, Tenente IM, Jacinto A, Ferreira MG (2013) Telomerase
541 Is Required for Zebrafish Lifespan. *PLoS Genet* 9(1).
542 doi:10.1371/journal.pgen.1003214.
- 543 30. Harley C, et al. (1992) Telomere length predicts replicative capacity of human
544 fibroblasts. *Proc Natl Acad Sci U S A* 89(21):10114–10118.
- 545 31. Renshaw SA, et al. (2006) A transgenic zebrafish model of neutrophilic inflammation.
546 *Blood* 108(13):3976–3978.
- 547 32. Ostrand-Rosenberg S (2008) Immune surveillance: a balance between protumor and
548 antitumor immunity. *Curr Opin Genet Dev*. doi:10.1016/j.gde.2007.12.007.
- 549 33. Loynes CA, et al. (2018) PGE2 production at sites of tissue injury promotes an anti-
550 inflammatory neutrophil phenotype and determines the outcome of inflammation
551 resolution in vivo. *Sci Adv*. doi:10.1126/sciadv.aar8320.

- 552 34. Freisinger CM, Huttenlocher A (2014) Live imaging and gene expression analysis in
553 zebrafish identifies a link between neutrophils and epithelial to mesenchymal
554 transition. *PLoS One*. doi:10.1371/journal.pone.0112183.
- 555 35. Heidenreich B, Rachakonda PS, Hemminki K, Kumar R (2014) TERT promoter
556 mutations in cancer development. *Curr Opin Genet Dev*.
557 doi:10.1016/j.gde.2013.11.005.
- 558 36. Feitsma H, Cuppen E (2008) Zebrafish as a cancer model. *Mol Cancer Res*.
559 doi:10.1158/1541-7786.MCR-07-2167.
- 560 37. Novoa B, et al. (2019) Rag1 immunodeficiency-induced early aging and senescence in
561 zebrafish are dependent on chronic inflammation and oxidative stress. *Aging Cell*.
562 doi:10.1111/accel.13020.
- 563 38. Maru GB, Gandhi K, Ramchandani A, Kumar G (2014) The Role of Inflammation in
564 Skin Cancer. *Inflammation and Cancer* doi:10.1007/978-3-0348-0837-8.
- 565 39. Tang L, Wang K (2016) Chronic inflammation in skin malignancies. *J Mol Signal*.
566 doi:10.5334/1750-2187-11-2.
- 567 40. Shalapour S, Karin M (2015) Immunity, inflammation, and cancer: An eternal fight
568 between good and evil. *J Clin Invest*. doi:10.1172/JCI80007.
- 569 41. White RM, et al. (2008) Transparent Adult Zebrafish as a Tool for In Vivo
570 Transplantation Analysis. *Cell Stem Cell*. doi:10.1016/j.stem.2007.11.002.
- 571 42. Cooper MS, et al. (2005) Visualizing morphogenesis in transgenic zebrafish embryos
572 using BODIPY TR methyl ester dye as a vital counterstain for GFP. *Dev Dyn*.
573 doi:10.1002/dvdy.20252.
- 574 43. Kishi S, et al. (2008) The identification of zebrafish mutants showing alterations in
575 senescence-associated biomarkers. *PLoS Genet* 4(8).
576 doi:10.1371/journal.pgen.1000152.
577
578

579 **Figure Legends**

580 **Figure 1. Short telomeres promote tumorigenesis in a non-cell autonomous manner. A)**

581 Experimental setup for the generation of zebrafish chimeras. Donor cells are transplanted
582 from a Tg(*mitfa:HRAS^{G12V}*; *β-actin:GFP*) embryo at the blastula stage into embryos resulting
583 from an incross of *tert*^{+/-}; Casper zebrafish. B) Representative images of adult chimera
584 zebrafish harboring melanoma in either WT or *tert*^{-/-} recipients. C) Melanoma occurrence
585 over time in chimeric fish. *tert*^{-/-} recipient fish have a higher risk of tumorigenesis than WT
586 recipient fish (p<0.05).

587 **Figure 2. *tert*^{-/-} tissues increase melanoma invasiveness and progression. A)**

588 of melanoma arising in a wildtype (upper panel) or *tert*^{-/-} recipient fish. Strong infiltration
589 into other tissues was typical in *tert*^{-/-} fish but not in wildtype (arrowheads). B) Melanoma

590 were staged according to histopathology into benign lesions (melanosis), non-invasive and
591 invasive malignant tumors. C) Analysis of malignant tumors for cellular atypia. Sample
592 numbers are indicated within the bars.

593 **Figure 3. G2 *tert*^{-/-} larvae with very short telomeres exhibit increased melanoma**
594 **micrometastasis.** A) Experimental design for melanoma allotransplants in zebrafish larvae.
595 Melanoma tumors were dissociated from *mitfa:HRAS; β-actin:GFP* zebrafish. HRAS
596 melanoma cells were then injected into blood circulation of 2dpf zebrafish larvae. Larvae
597 were kept in embryo medium for 7 days post injection (7dpi). B) Representative images of
598 HRAS melanoma cells spread (green) in WT or G2 *tert*^{-/-} larvae at 7dpi. C) Time-course of
599 HRAS melanoma cells spread in a group of WT and G2 *tert*^{-/-} larvae (p<0.01 at 7dpi, WT
600 N=10 and G2 *tert*^{-/-} N=11). D) Melanoma tumors are more invasive in G2 *tert*^{-/-} larvae
601 (p=0.0205, WT N=32 and G2 *tert*^{-/-} N=31). A linear regression of three time-points (1, 4 and
602 7 dpi) was used to calculate the slope of melanoma invasiveness. Each dot represents one
603 larvae allotransplant.

604 **Figure 4. Telomerase deficient tissues present higher levels of senescence and**
605 **inflammation and modulate their environment.** A, B, C) RT-qPCR analysis comparing the
606 expression levels of *ink4ab* (p16/15), *cdkn2a* (p21) and *tnfa* (TNF) of 4dpf WT and G2 *tert*^{-/-}
607 larvae and 9month WT and *tert*^{-/-} adult intestine tissue (* p<0.05, ** p<0.01 N=30). D)
608 Representative images of SA-β-Gal assay comparing WT and G2 *tert*^{-/-} 4dpf zebrafish
609 embryos. Yolk sack staining is nonspecific. E) Scheme for generating chimeras in which G2
610 *tert*^{-/-} blastula cells are transplanted into WT embryos (G2 *tert*^{-/-}→WT). SA-β-Gal assay
611 showing increased senescence in 4dpf WT embryos with injection of G2 *tert*^{-/-} cells. F)
612 Scheme of G2 chimeras generation where WT or G2 *tert*^{-/-} blastula cells are transplanted into
613 WT Tg(*mpx:GFP*) embryos carrying labelled neutrophils with green fluorescent protein:
614 WT→WT Tg(*mpx:GFP*) vs. G2 *tert*^{-/-}→WT Tg(*mpx:GFP*). Representative images of the

615 chimeras at 4dpf, neutrophils are represented at green; G) Quantification of neutrophils at
616 4dpf. Non-injected Tg(*mpx:GFP*) were used as controls. Each data point represents one
617 zebrafish (** $p < 0.01$, non-injected N=24, WT N=33 and TERT N=25).

618 **Figure 5. Increased tumor invasiveness in G2 *tert*^{-/-} larvae is rescued by inhibiting**
619 **inflammation.** A) Allotransplants of primary tumor cells extracted from melanoma in adult
620 fish into 2dpf larvae that were kept in embryo medium containing Aspirin or Celecoxib
621 (COX-2 selective inhibitor). B) Representative images of melanoma invasiveness at 7dpi
622 upon Aspirin treatment. C) Time-course of melanoma invasiveness in WT and G2 *tert*^{-/-}
623 larvae under Aspirin treatment. D) Slope of HRAS melanoma spread between 1, 4 and 7dpi.
624 Comparison of invasiveness in a WT or G2 *tert*^{-/-} either untreated (Control) or containing
625 Aspirin (WT N=26 and G2 *tert*^{-/-} N=29) or Celecoxib (WT N=19 and G2 *tert*^{-/-} N=13). Each
626 dot represents one zebrafish larva from 2-3 biological replicates.

627 **Supplementary Figure 1. *tert* genetic status of chimera recipients does not influence the**
628 **number of melanocytes in larvae or adults.** A) Representative images of 3dpf chimeras
629 exhibiting high (left) and low (right) number of melanocytes. Blastula *mitfa:HRAS*; β -
630 *actin:GFP* cells were injected into the same stage embryos resulting from an incross of *tert*^{+/-}
631 ; Casper. Larvae were genotyped at 3dpf and followed individually until 11dpf. B) No
632 significant differences can be observed between the number of melanocytes in hosts of
633 different *tert* genotype, both at 3dpf and 11dpf. Each point in the graph represents an
634 individual animal. Data are represented as mean \pm SEM. C) Chimeras harboring a tumor
635 were analyzed for extent of pigmentation in adults. Left side animals with high pigmentation,
636 right side: low pigmentation. C) Quantification of pigmented area given as in percent of total
637 surface. Each datapoint represents one animal (both sides). Pigmented area did not
638 significantly differ depending on the host genotype. Data are represented as mean \pm SEM.

639

Figure 1

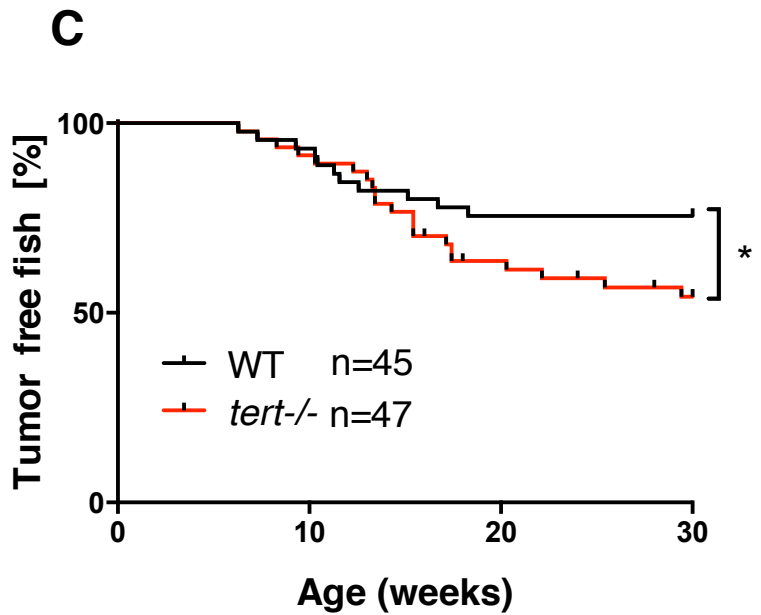
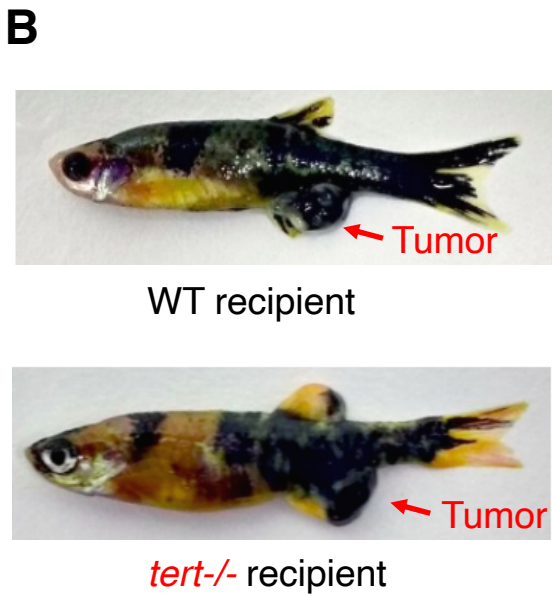
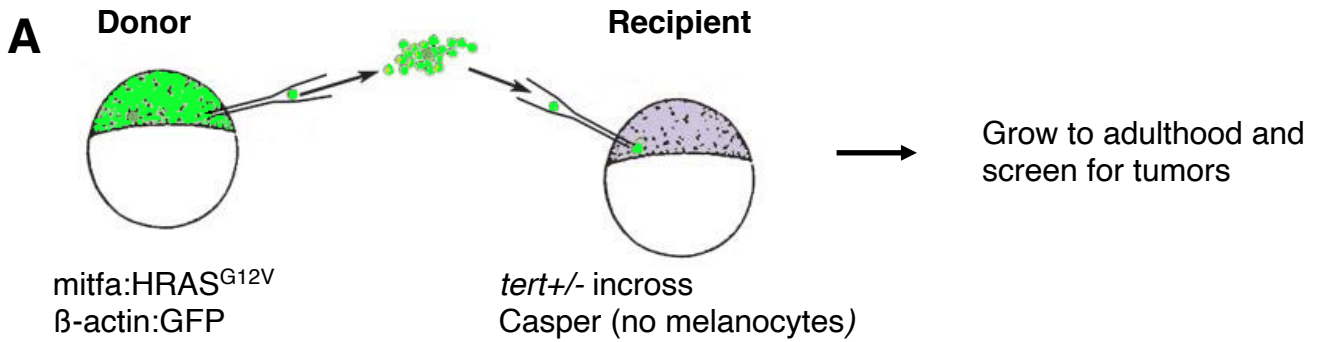
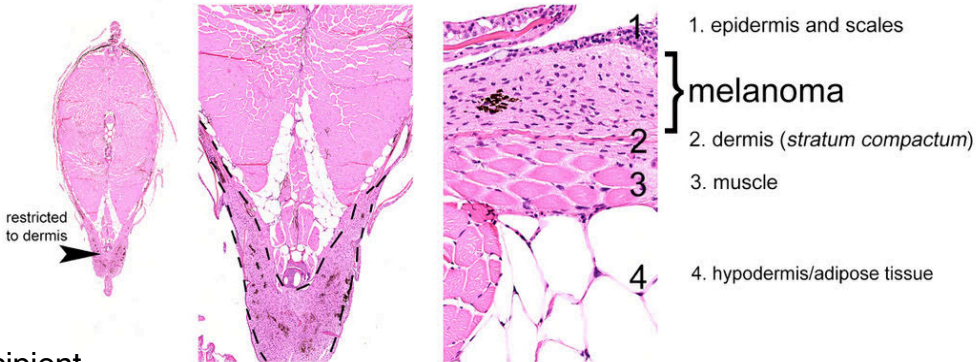


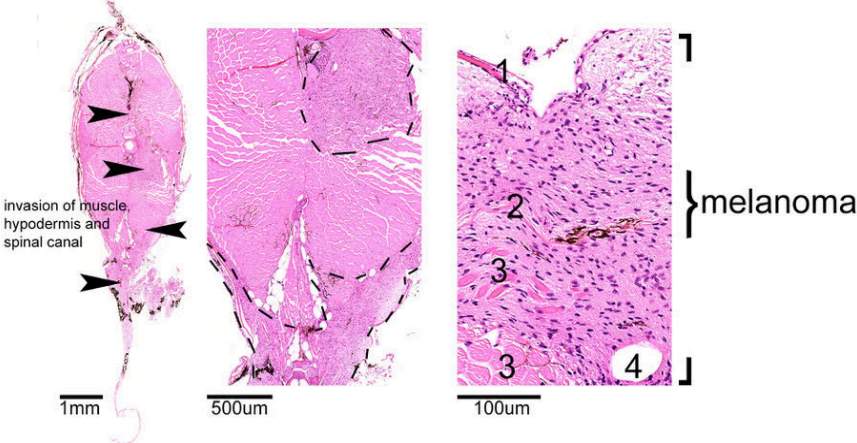
Figure 2

A

WT recipient

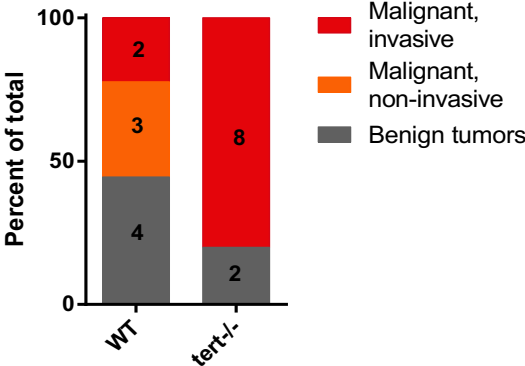


tert^{-/-} recipient



B

Tumor staging



C

Cellular atypia

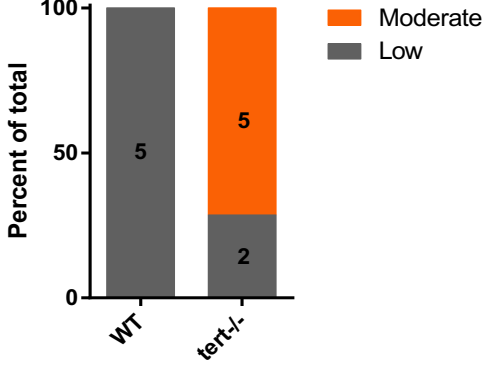
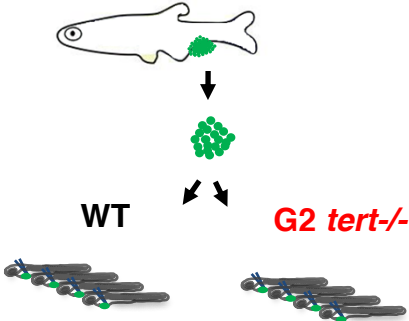
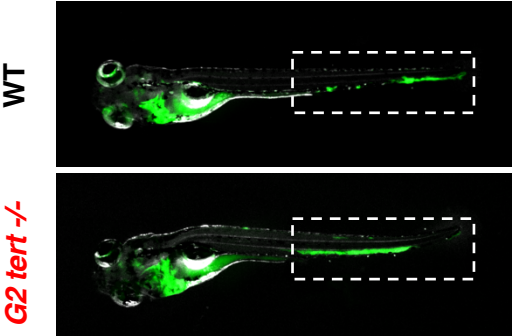


Figure 3

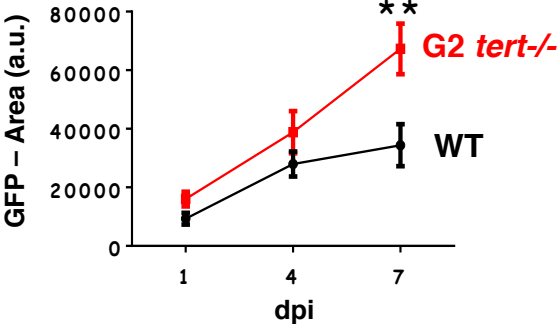
A



B



C



D

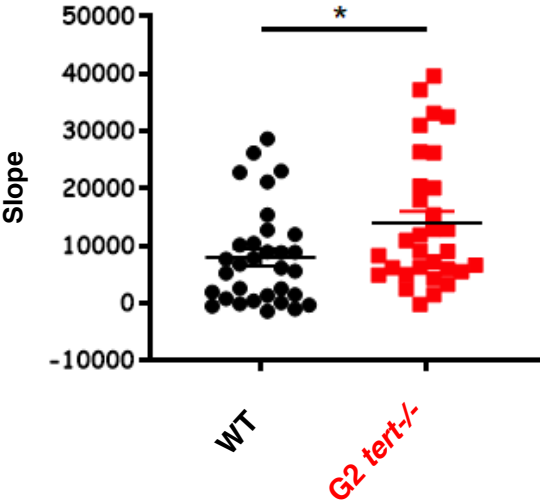


Figure 4

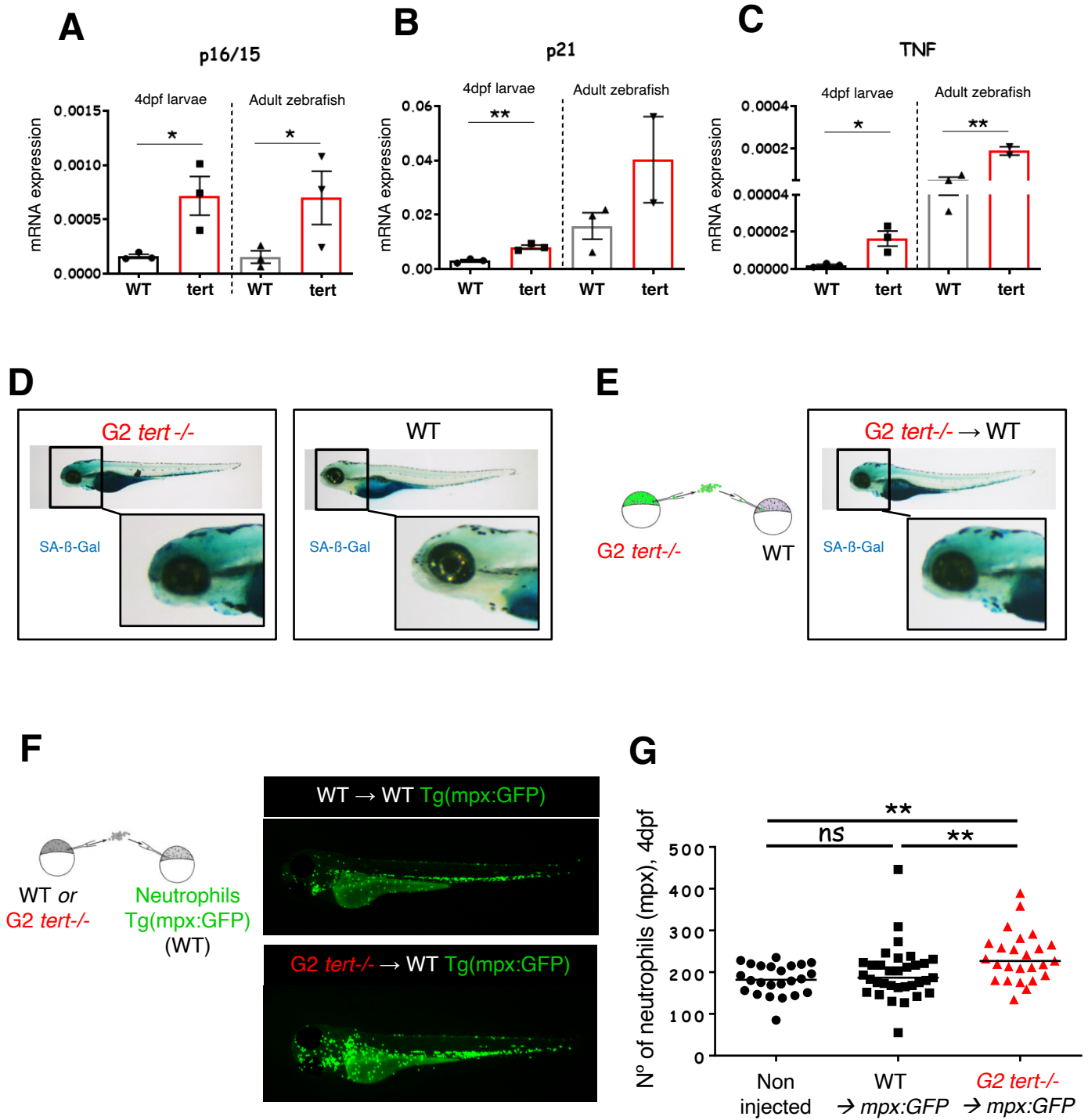
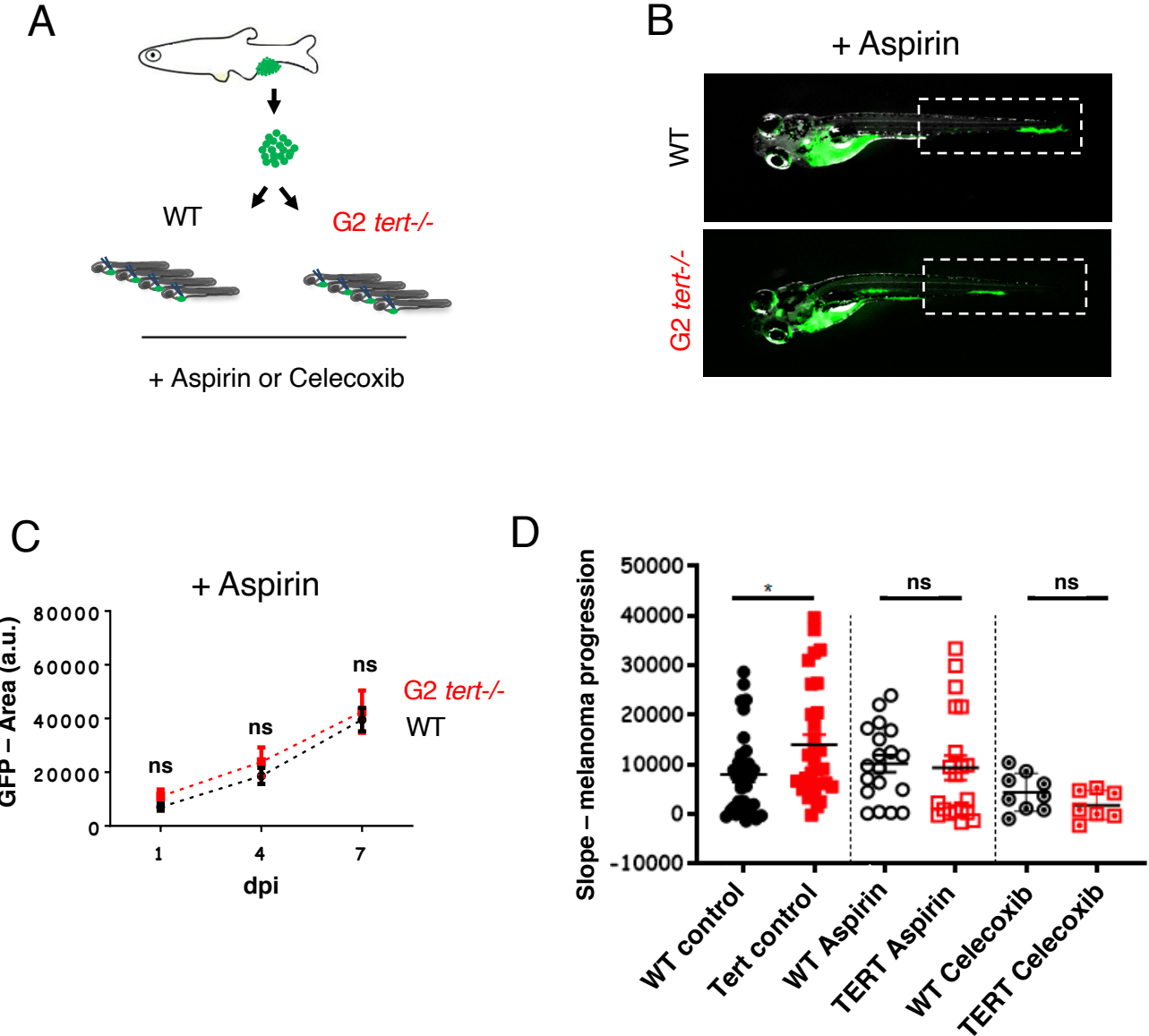


Figure 5



Supplementary Figure 1

